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ORIGINAL ARTICLE

# Antiproliferative activities of auraptenol against drug-resistant human prostate carcinoma cells are mediated via programmed cell death, endogenous ROS production, and targeting the JNK/p38 MAPK signal pathways

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## Summary

***Purpose:*** *Prostate cancer is considered to be one of the most common cancers in men and as such there is a pressing need for finding new therapeutic agents to treat this disease. Therefore, the main purpose of the current research work was to study the anticancer effects of a naturally occurring coumarin- Auraptenol- against drug-resistant human prostate cancer cells and evaluate its effects on programmed cell death, reactive oxygen species (ROS) production, and JNK/p38 MAPK signalling pathway.*

***Methods:*** *Cell proliferation was examined by CCK8 cell viability assay. Apoptosis-related studies were checked by fluorescent microscopy using acridine orange (AO)/ethidium bromide (EB) and Hoechst staining, as well as flow cytometry using annexin V/propidium iodide (PI) assay. Western blot was used to study the effects of Auraptenol on apoptosis-related protein expressions including Bax, Bcl-2, as well as JNK/p38 MAPK signalling pathway. ROS production was evaluated by flow cytometry.*

***Results:*** *The results showed that Auraptenol caused significant reduction in the viability of the human LNCaP prostate carcinoma cells in a dose-dependent manner, exhibiting an $IC_{50}$ of 25 μM in cancer cells and $IC_{50}$ of 100 μM in normal PNT2 cells. The AO/EB staining assay showed that Auraptenol inhibited the viability of cancer cells via induction of apoptotic cell death, which was associated with increase in Bax and decrease in Bcl-2 levels. Hoechst staining results also confirmed that Auraptenol induced programmed cell death. The apoptotic cells increased from 0.8% in the control to 32.5% in the study group at 50 μM concentration of Auraptenol. Auraptenol also induced an increase in ROS production in a dose-dependent manner. Finally, this molecule blocked the JNK/p38 MAPK signal pathway concentration-dependently in human prostate cancer cells.*

***Conclusion:*** *In conclusion, the current study indicates that this molecule could be developed as a potential anticancer drug against human prostate carcinoma provided further studies are carried out.*

***Key words:*** *auraptenol, prostate cancer, apoptosis, microscopy, drug-resistant*

## Introduction

Accounting for 33% of all the newly detected cancer in men, prostate cancer is one of the leading malignancies in United States in males [1]. As per estimates 0.22 million people were diagnosed with prostate cancer and around 2900 died of it in 2003 [2,3]. Although prostate cancer shows geographical variations, the incidence is higher in United States, Scandinavia and Canada compared to Asian countries [4]. Over the last few decades, the incidence of prostate cancer has increased significantly. Despite

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its higher mortality, its etiology is still not well understood [5]. The treatment for prostate cancer involves surgery, chemotherapy hormonotherapy and/or radiotherapy. Nonetheless, prostate cancer frequently relapses and develop drug-resistance, thus becoming difficult to manage [6]. Therefore, the development of new therapeutic agents for prostate cancer is needed for its efficient management. In recent years, the utilization of highly active components of edible medicinal plants as gained considerable importance. Additionally, great amount of research is directed at screening plant-derived compounds and to generate efficient derivatives of these compounds through semi-synthetic approaches [7]. Out of plant-derived secondary metabolites, coumarins have attained huge importance for their anticancer effects [8]. Auraptenol is an important coumarin generally extracted from bitter (Seville) orange oil. It has been shown to exhibit a wide array of pharmacologically properties such as antidepressant and antitumor [9,10]. Nonetheless, there is not a single report on the anticancer effects of Auraptenol on prostate cancer cells.

This study, henceforth, was designed to investigate the anticancer effects of Auraptenol against human prostate cancer cells and to explore the underlying mechanism of its anticancer effects. JNK/p38 MAPK signalling pathway is one of therapeutically important pathways that has been shown to be activated in a wide array of cancer cell types [11]. In this study, the effects of Auraptenol were also investigated on this signalling pathway as well. The main purpose of this research was to study the anticancer effects of a naturally occurring coumarin-Auraptenol- against drug-resistant human prostate cancer cells and evaluate its effects on programmed cell death, reactive oxygen species (ROS) production, and JNK/p38 MAPK signalling pathway.

## Methods

*Cell viability assay*

The drug-resistant LNCaP prostate cancer cell line and the normal cell line PNT2 were treated with different concentrations of Auraptenol and cultured in 96-well plates for 24 h. Afterwards, 10µl CCK-8 (Dojindo Laboratories, Kumamoto, Japan) was added to the culture plates, which were incubated for 2h at 37°C in humidified 95% air and 5% $CO_2$. The absorbance was measured at 450 nm using a microplate Reader (Bio-Rad, Hercules, CA, USA).

*AO/EB and Hoechst staining*

The LNCaP cells ($0.6\times10^6$) were cultured in 6-well plates and treated with Auraptenol at concentrations of 0, 12.5, 25 and 30 µM for 24 at 37°C. Subsequently, 25 µl of cell culture were put onto glass slides and stained with AO/EB or Hoechst, separately. The slides were then cover-slipped and examined under fluorescence microscope.

*Annexin V/PI staining*

ApoScan kit was used to determine the apoptotic LNCaP cell percentage. In brief, Auraptenol-treated LNCaP cells ($5\times10^5$ cells per well) were incubated for 24 h. This was followed by staining of these cells with annexin V-FITC/PI. The percentage of apoptotic LNCaP cells at each concentration was then determined by flow cytometry.

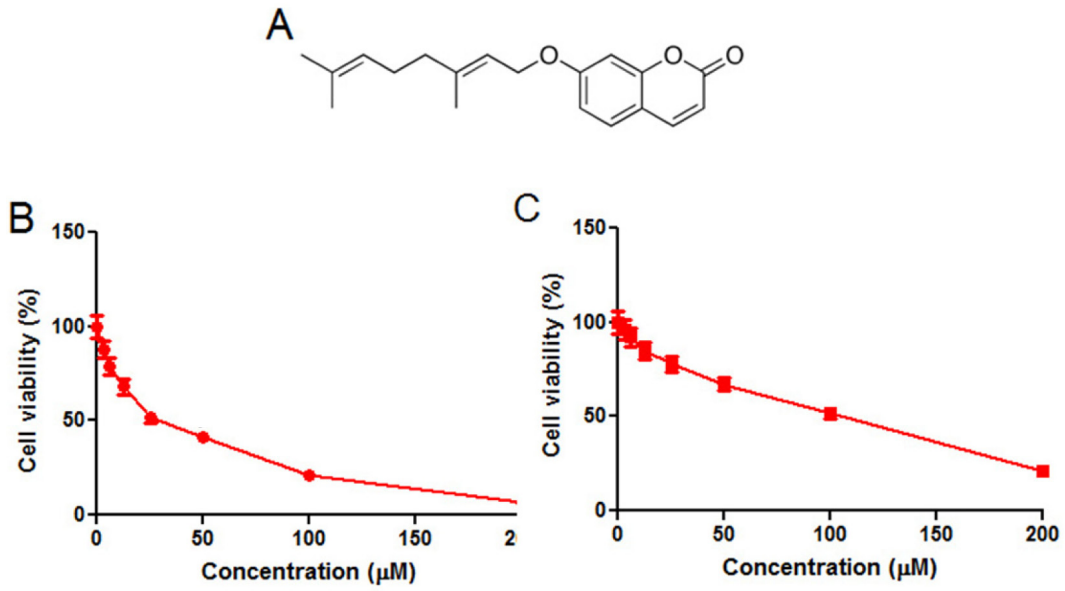


**Figure 1. A:** Structure of auraptenol. Effect of auraptenol at different concentration on the viability of **(B)** drug-resistant LNCaP prostate cancer cells and **(C)** on the normal prostate PNT2 cells. The experiments were performed in triplicate ($p<0.01$). $P<0.01$ was considered as statistically significant and data was presented as mean±SEM.

*Determination of ROS level*

The LNCaP cells were firstly cultured in 6-well plates at a cell density of $1.5\times10^5$. This was followed by subjecting the cells to 0, 12.5, 25 and 50 μM Auraptenol treatment at 37°C. The cells were thereafter washed with phosphate buffered saline (PBS) and dihydrofluorescein diacetate (500 μl) for 30 min in the dark for the determination of ROS using flow cytometry.

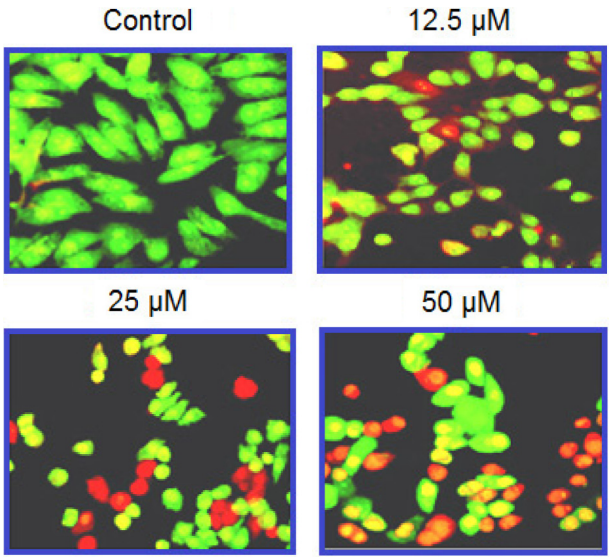


**Figure 2.** AO/EB staining showing the induction of apoptosis in LNCaP prostate cancer cells at indicated concentrations of auraptenol. The Figure shows green fluorescence in untreated cells and orange/yellow fluorescence in auraptenol-treated cells indicating onset of apoptosis. The experiments were performed in triplicate.

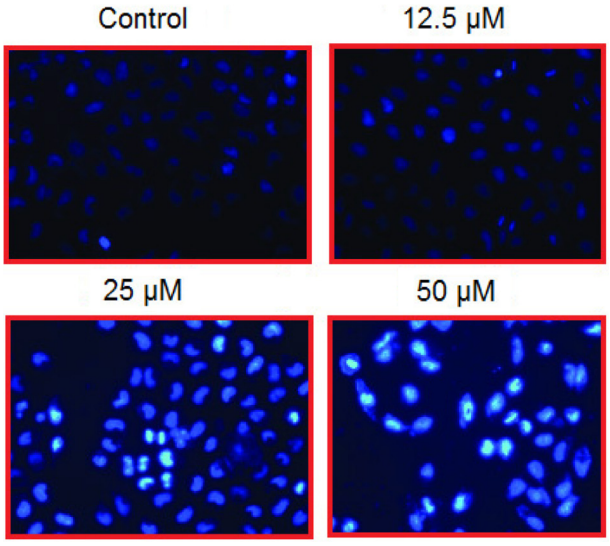


**Figure 3.** AO/EB staining showing the induction of apoptosis in LNCaP prostate cancer cells at indicated concentrations of auraptenol. The Figure shows that auraptenol-treated (0, 12.5, 25 and 50 μM) cells showed chromatin condensation and nuclear fragmentation. The experiments were performed in triplicate.

*Western blot analysis*

The LNCaP cells were exposed to varying Auraptenol doses prior to lysing with lysis buffer. Twenty μg of total protein quantified by performing BCA assay were subjected to 8% SDS-PAGE gel. Resolving of the protein content was accomplished electrophoretically. Afterwards, proteins were loaded to PVDF membranes followed by primary antibody treatment. Thereafter, membranes were subjected to secondary antibodies overnight at 4°C. Finally, the protein signals were detected and visualized via ECL (enhanced chemiluminescence). Actin was used as control for normalisation.

*Statistics*

The results are presented as mean±standard deviation from three independent experiments. Differences between the groups were examined by Student's *t*-test using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate statistically significant difference.

## Results

*Auraptenol suppresses the proliferation of prostate cancer cells*

To find out if Auraptenol suppresses the proliferation of the drug-resistant LNCaP cells, CCK-8 cell viability assay was performed. This revealed that upon treatment with Auraptenol (Figure 1A) the proliferation rate of drug-resistant LNCaP cells was remarkably decreased. An $IC_{50}$ of 25 μM was observed for Auraptenol against the LNCaP cells (Figure 1B). To assess the toxicity of Auraptenol,

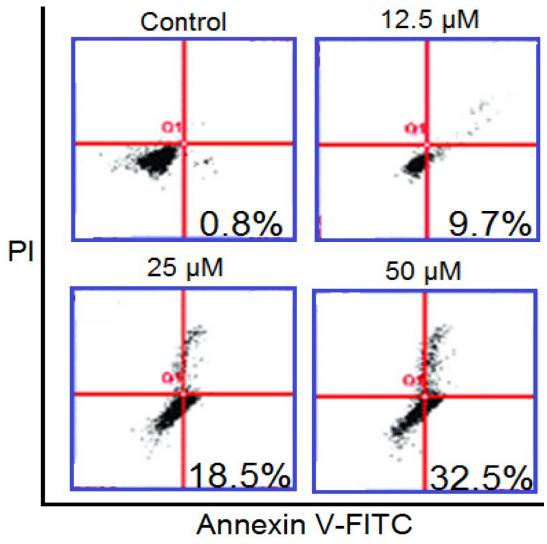


**Figure 4.** Annexin V/PI staining showing the percentage of apoptotic LNCaP cells at indicated concentrations of auraptenol. The auraptenol-treated cells (0, 12.5, 25 and 50 μM) showed increasing percentage of apoptotic cells with increasing doses of auraptenol. The experiments were performed in triplicate.

its effects were investigated on the normal PNT2 cells and the results showed Auraptenol exerted comparatively lower toxicity against the normal PNT2 prostate cells as evidenced from the $IC_{50}$ of 100 µ (Figure 1C).

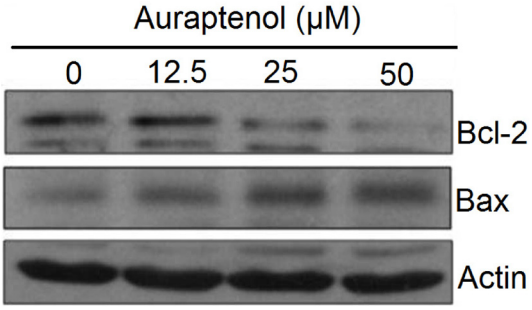


**Figure 5.** Western blot analysis showing the effect of auraptenol on the expression of Bax and Bcl-2. The Figure shows increasing expression of Bax and decreasing expression of Bcl-2. The experiments were performed in triplicate.

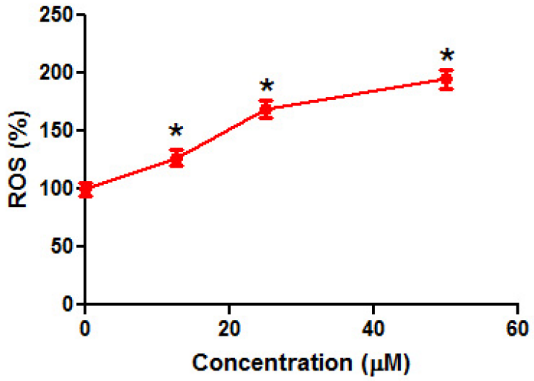


**Figure 6.** Estimation of ROS levels at indicated concentrations of auraptenol in LNCaP prostate cancer cells by flow cytometry. The Figure shows increasing ROS levels with increasing auraptenol doses. The experiments were performed in triplicate (*p<0.01).

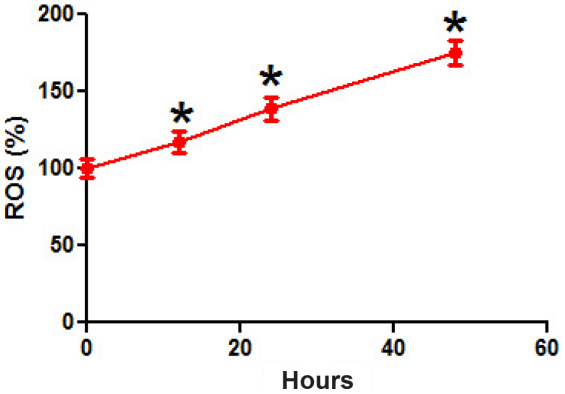


**Figure 7.** Estimation of ROS levels at 25µM of auraptenol and indicated time intervals in LNCaP prostate cancer cells by flow cytometry. The Figure shows auraptenol induced time-dependent ROS production at 25 µM dose. The experiments were performed in triplicate (*p<0.01).

*Apoptosis induction by Auraptenol in LNCaP cells*

The apoptosis in the Auraptenol-treated LNCaP cells was assessed by AO/EB and Hoechst staining. The AO/EB staining revealed that Auraptenol caused increase in the orange colored cells, suggestive of apoptosis (Figure 2). The Hoechst assay showed that Auraptenol caused nuclear fragmentation of the drug-resistant LNCaP prostate cancer cells, confirming their autophagic cell death (Figure 3). Annexin V/PI staining showed that the apoptotic LNCaP cell percentage increased to about 32.5% at 50 µM concentration of the molecule compared to approximately 0.8% in the untreated LNCaP cells (Figure 4).

*Auraptenol affects the Bax and Bcl-2 expression*

The effects of Auraptenol were also examined on the expression of Bax and Bcl-2 in LNCaP drug-resistant prostate cancer cells by western blotting. The LNCaP cells were treated with 0, 12.5, 25 and 50 µM concentrations of Auraptenol and then the proteins were extracted. SDS-PAGE was run to examine the expression of Bax and Bcl-2. The results showed that the expression of Bax was increased while that of Bcl-2 was decreased in a concentration-dependent manner (Figure 5).

*Auraptenol causes generation of ROS*

Flow cytometry was used to determine the ROS levels in LNCaP drug-resistant prostate cancer cells at 0, 12.5, 25 and 50 µM concentrations and the results showed that the ROS levels increased concentration-dependently. The ROS levels increased from 100% in the control to about 197% at 50 µM of Auraptenol (Figure 6). The effects of Auraptenol concentration (12.5 µM) were also assessed at 0, 12, 24 and 48 h time intervals and the

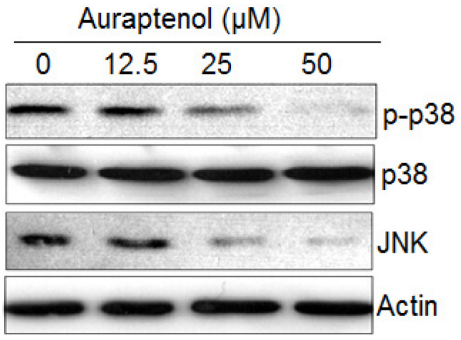


**Figure 8.** Western blot analysis showing the effect of auraptenol on the JNK/P38 MAPK signalling pathway. Auraptenol led to inhibition of p38 phosphorylation dose-dependently while no effects were seen on the total MAPK expression. The expression of JNK was also suppressed in a dose-dependent manner. The experiments were performed in triplicate.

results showed that the ROS levels also increased in a time-dependent manner (Figure 7).

*Auraptenol inhibits the JNK/p38 MAPK signalling pathway in LNCaP cells*

The effects of Auraptenol were also investigated on the JNK/p38 MAPK signalling pathway and the results of the Western blot analysis showed that this molecule inhibited the phosphorylation of p38 in a dose-dependent manner (Figure 8). Nonetheless, no effects were observed on the total MAPK expression. Moreover, the expression of JNK was also suppressed in a concentration-dependent manner.

## Discussion

Prostate cancer is one of the most prevalent malignancies in males world-over and causes significant morbidity and mortality. The incidence of prostate cancer is increasing at an alarming rate and hence identification of novel therapeutic agents is the need of the hour [12]. Molecules derived from plants and microbes are believed to serve as lead molecules for the generation of effective therapy of different types of cancers including prostate cancer [13]. Herein, the main purpose of the current research was to study the anticancer effects of a naturally occurring coumarin - Auraptenol - against drug-resistant human prostate cancer cells and evaluating its effects on programmed cell death, ROS production, and JNK/p38 MAPK signalling pathway. The results of this study revealed that Auraptenol caused significant reduction in the viability of the human LNCaP prostate carcinoma cells in a dose-dependent manner, exhibiting an $IC_{50}$ of 25 μM in cancer cells. These observations are also consistent with previous studies wherein coumarins have been shown to suppress the proliferation of cancer cells. For example 7-hydroxycoumarin has been shown to suppress the proliferation of the human lung cancer cells [14]. Similarly, Umbelliprenin has been shown to inhibit the growth of melanoma cells [15]. Previous studies have shown that coumarins exert anticancer effects via multiple mechanisms and one of the important mechanism is the induction of apoptosis. For instance Xanthoxyletin, a naturally occurring coumarin, has been shown to induce apoptosis in human gastric adenocarcinoma cells [16]. Consistently, we also carried out different assays to examine if Auraptenol induces apoptosis in the LNCaP prostate cancer cells. The AO/EB staining assay showed that Auraptenol inhibits the viability of cancer cells via induction of apoptotic cell death, which was associated with increase in Bax and decrease in Bcl-2 levels. Hoechst staining results also confirmed that the molecule induced programmed cell death. The apoptotic cells increased from 0.8% in the control to 32.5% at 50 μM concentration of Auraptenol as evidenced from the Annexin V/PI staining. Many plant-derived metabolites have been shown to induce apoptosis in cancer cells via production of substantial amounts of ROS in cancer cells. For example, Sulforaphane triggered apoptosis in prostate cancer cells via generation of ROS [17]. Therefore, we also examined if Auraptenol induced apoptosis in LNCaP prostate cancer cells via production of ROS. What we found was that this molecule caused generation of significant amounts of ROS in LNCaP prostate cancer cells both concentration- and time-dependently. The effect of the molecule was also examined on the therapeutically important JNK/p38 MAPK pathway [11] and the results showed that Auraptenol also blocked the JNK/p38 MAPK signalling pathway concentration-dependently in human prostate cancer cells, indicative of the anticancer potential of Auraptenol.

## Conclusion

The findings of the current study revealed that Auraptenol coumarin compound inhibits the growth of prostate cancer cells via induction of ROS-mediated apoptosis and has the potential to be developed as a potential anticancer drug against human prostate carcinoma provided further studies are carried out.

## Conflict of interests

The authors declare no conflict of interests.

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